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Fractionation of whey proteins by means of membrane adsorption chromatography

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Abstract

The separation of different major and minor proteins from acid whey with membrane adsorption chromatography at lab scale was investigated. Macroporous membranes with strong anionic or cationic ligands for fast operation (Sartobind Q and S nano) were used in order to bind and subsequently elute the proteins. Food grade buffers were applied for equilibration and elution steps. Desorption was realized by ionic strength gradient with sodium chloride. A two step process was developed where the six target proteins could be efficiently isolated. For maximum exploitation of membrane capacity as well as for highest purity of the various protein fractions potential displacement effects on both anion and cation exchanger have been investigated.

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Keywords: whey proteins; membrane adsorption chromatography; displacement effects.

1. Introduction

The issue of the presented work is the separation and purification of valuable proteins from cheese whey. A novel process for high throughput ion exchange chromatography (IEC) is investigated. The applied method is called membrane adsorption chromatography (MAC). It is technologically and economically advantageous compared to the commonly applied packed bead chromatography. IEC is generally well suited for the separation of proteins if they provide a wide range of isoelectric points (IEP). Thus, different net charges dependent on ambient pH can be used for selective ad- and desorption to anionic or cationic stationary phases.

Native whey, which is a byproduct of cheese production, contains valuable ingredients, such as proteins and lactose. Worldwide only a small percentage (<5%) of the generated cheese whey is used for

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further applications (e.g. WPI- and WPC-production) while the remaining cheese whey is wasted or used as fertilizer. A wide range of bio and techno functional applications is possible regarding the proteins in whey [1]. Amongst them are the two major whey proteins α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) as well as the minor protein fractions lactoferrin (Lf), immunoglobulins (Ig), lactoperoxidase (LPO) and bovine serum albumin (BSA). Some of the mentioned proteins have been isolated from whey by means of membrane filtration or chromatographic processes but in both cases compromises are necessary. In any case only a few proteins are separated from the protein mixture [2, 3, 4]. Often the degree of denaturation is quite high due to thermal or acidic treatments in membrane processes. For the application of whey protein fractions in functional foods or for technological reasons the isolation of native proteins is desired. Therefore, separation with MAC technology is a fast and gentle method which has already been investigated for the fractionation of several whey proteins. In the work of Plate et al. [5] the isolation of lactoferrin and lactoperoxidase by cation exchange membrane chromatography was studied resulting in highly pure fractions of Lf and LPO (88 and 95% purity, respectively). The adsorption behavior of β-Lg, α-La and BSA was investigated and resulted in a higher affinity of β-Lg to strong anion exchangers compared to BSA and α-La [6].When binding capacity was saturated a β-Lg fraction with ≥99% purity could be obtained. Under different experimental conditions adsorption of β-Lg, α-La and BSA on strong anion exchangers was observed by Splitt et al. [7]. The proteins have been eluted in different fractions although no baseline separation was possible. The objective of this work is the development of a process with coupled anion and cation exchange membranes for the selective purification of the proteins α-La, β-Lg, BSA, IgG, Lf and LPO from acid whey under non-denaturing conditions.

2. Materials and Methods

2.1. Chemicals

For IEC sodium phosphate (Na2HPO4) was obtained from Sigma-Aldrich (Steinheim, Germany), sodium acetate and sodium chloride were purchased from Merck chemicals (Darmstadt, Germany). For HPLC buffers trifluoroacetic acid (TFA) from Pierce Chemicals (Bonn, Germany) and acetonitrile from Sigma-Aldrich (Steinheim, Germany) were used. All reagents were analytical grade. All buffers and solutions were prepared using bidistilled water (Millipore, Schwalbach, Germany). Buffers were vacuum-filtered using 0.2 µm RC 58 membrane filters (Whatman, Dassel, Germany). Whey samples were filtered using 0.45 µm disposable syringe filters (Macherey-Nagel, Düren, Germany). Protein standards of α-La, β-Lg, BSA, LPO, Lf and IgG were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Preparation of whey

Pasteurized skim milk was obtained from a local dairy. Acid whey was prepared using lactic acid bacteria Flora danica at 26°C. At pH 4.6 the acid whey was separated from the curd through a curd cloth. Freshly prepared whey was stored in aliquots at -37°C and thawed in a water bath at 50°C on the day of use. For all experiments the same batch of whey served as sample. Before sample injection into the IEC system, phospholipids were removed according to the method described by Maubois et al. [8] to prevent blocking of the adsorber membranes. Consequently, whey samples were adjusted to the pH of the applied buffers and then passed through a syringe filter. The protein content of both types of whey is shown in Table 1.

Table 1. Protein concentrations of pre-treated acid whey and IEPs of whey proteins [9, 10, 11]

Protein fraction	Acid whey (mg·ml^{-1})	IEP
α-Lactalbumin	980	4.2-4.5
β-Lactoglobulin	3410	5.35-5.41
BSA	180	5.13
Lactoferrin	170	8.8-9.5
Immunoglobulin G	360	5.5-8.3
Lactoperoxidase	87	9.0
Total protein	5459	

2.3. Ion exchange chromatography

Sartobind Q nano 3 ml (Q-MA) and Sartobind S nano 3 ml (S-MA) units were obtained from Sartorius Stedim (Göttingen, Germany). The IEC instrumentation for the fractionation process was an Äkta Basic System (GE Healthcare, München, Germany) containing an UV-detector UV900 with a 2 mm flow cell, an electrical conductivity detector (0–100 mS·cm^{-1}) and a pH-detector pH/C-900, a sample pump P960 and a fraction collector Frac-950. Fractionation was carried out according to the following steps: 1) Equilibration of membrane adsorbers with buffer A, 2) Sample injection, 3) Wash out unbound sample with buffer A, 4) Elution of bound proteins by NaCl-gradient with buffers A and B, 5) Reequilibration of membrane adsorbers with buffer A. Buffer A: 0.1 M sodium acetate (NaAc) or 0.03 M sodium phosphate (NaPhos); buffer B: 1 M NaCl added to buffer A. The experimental conditions were 20°C ambient temperature, 12 ml·min^{-1} flow rate at approx. 0.8 MPa system pressure and peak detection at 226 nm wavelength.

2.4. RP-HPLC

The quantitative determination of whey proteins in whey samples and fractions obtained in IEC was done by RP-HPLC. A method was developed for the simultaneous analysis of α-la, β-Lg, BSA, IgG, LPO and Lf using a PLRP-S 300Å 8µm column, 150 x 4,6 mm (Latek, Eppelheim, Germany). Eluent A contained 0.1% (v/v) TFA dissolved in water and eluent B was a mixture of 0.0555% (v/v) TFA, 80% (v/v) acetonitrile and 20% (v/v) water, the flow rate was 1 ml·min^{-1} and temperature was 40°C. Peak detection was at 226 nm for all proteins except for LPO. LPO was detected at 414 nm. The gradient started with 43% B for 4 min and was then increased to 47% within 8 min, to 52% during 3 min, to 57% within 15 min and to 90% after 1 min and the final step was 100% B after 1 min. The last step was kept for 30 s and then the gradient returned to starting conditions within another 30 s.

Peak integration gives protein concentration (mg·ml^{-1}) of the single proteins in each eluted fraction, in the substrate and in the effluent (referred to IEC). From the protein concentration recovery and purity can be calculated for each protein and fraction. Recovery (%) of one protein is the ratio of protein concentration in a peak fraction and in the substrate. Purity (%) of one protein in an elution fraction is the ratio of protein concentration and total protein concentration.

3. Results and Discussion

Binding of whey proteins to anion and cation exchange membranes was investigated under varied conditions in terms of buffer system and pH. The two buffer systems were 0.1 M sodium acetate (NaAc)

and 0.03 M sodium phosphate (NaPhos). For NaAc the pH-range was 4.0-5.7 and for NaPhos pH 6.4-7.4. Basically, proteins of interest must be bound to the stationary phases so that they can be eluted separately. Therefore, a starting pH has to be found where most proteins bind completely. After their adsorption a separate elution is required for the isolation of single protein fractions. Stepwise elution was carried out by increasing ionic strength (0-1 M NaCl). The fractionation process is divided into two main steps: ad- and desorption of whey proteins with low IEP on a strong anion exchange membrane (Q-MA) and subsequently ad- and desorption of proteins with high IEP on a strong cation exchange membrane (S-MA).

3.1. Binding of whey proteins to anion exchange membranes

A Q-MA is used to bind negatively charged proteins. To assure a negative net charge of proteins the chosen pH for whey and buffers is as high as possible. Therefore NaPhos with a pH-range of 6.4-7.4 (steps of 0.2 pH-units) is applied for the experiments. Starting pH is scouted in order to find the optimum pH for complete binding of proteins and their separate elution as far as possible. For stepwise elution NaCl-concentration set-up was 0.2, 0.5 and 1 M NaCl. Proteins assumed to bind on Q-MA in the tested pH-range are α-La, β-Lg, BSA and partly IgG (for IEPs see Table 1).

Throughout the scouted pH-values β-Lg and BSA adsorbed completely and did not occur in the effluent. This result was expected due to their IEPs. Surprisingly, almost no α-La adsorbed to the cationic ligands. Small amounts of α-La and IgG interacted with the stationary phase but eluted immediately before the ionic strength was increased. Hence, α-La and IgG were collected in the effluent together with LPO and Lf. BSA and β-Lg eluted as soon as the ionic strength was increased. BSA eluted in a first peak at approx. 0.1 M NaCl, β-Lg eluted in the same peak or afterwards at approx. 0.2 M NaCl. No protein desorption was observed at NaCl-concentrations above 0.2 M NaCl.

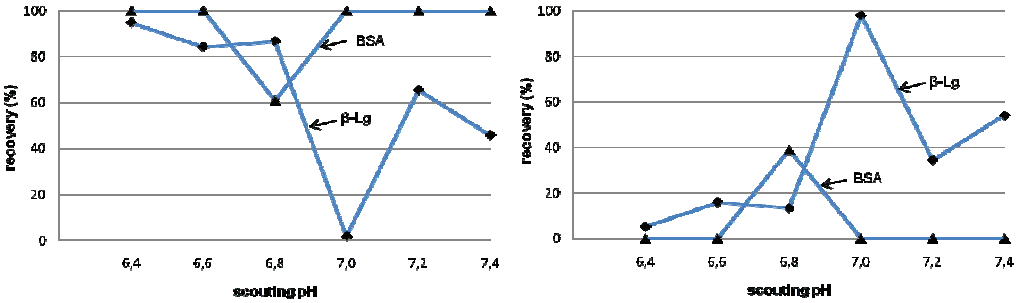

Fig. 1. (a) Recovery of β-Lg and BSA in the first elution peak at 0.1 M NaCl; (b) in the second elution peak at 0.2 M NaCl in dependence of buffer pH

Comparing the separation of BSA and β-Lg influenced by buffer pH, recovery of both proteins in the first and second peak is shown in Figure 1. BSA was completely found in the first peak throughout the experiments except of pH 6.8 (see Figure 1 (a)). Here only 60% of the total amount appeared in the first peak and 40% eluted with the second peak (Figure 1 (b)). The elution of BSA seemed to be quite independent from buffer-pH. Desorption of β-Lg was observed to be more influenced by buffer-pH. The higher the pH-value the less β-Lg was eluted at low ionic strength. Expressed as recovery of β-Lg in one peak it is obvious that at lower pH 80-95% eluted in the first peak and at pH > 7 less than 70% eluted in the first peak. Best separation occurred at pH 7, here BSA was completely eluted at 0.1 M NaCl and β-Lg was entirely eluted at 0.2 M NaCl.

As expected, BSA and β-Lg adsorbed to the anion exchange membrane and a quite satisfying separation of those two proteins was possible. A very poor binding of α-La was observed which was not expected due to its IEP and associated net charge. The same result was found by Goodall et al. [6]. With increasing amount of whey protein injected into the Q-MA more β-Lg and less BSA and α-La were bound. Thus, β-Lg displaced the other protein fractions. This might be explained by different affinities of the charged proteins to the stationary phase. While β-Lg and BSA have similar net charges at pH 7, the net charge of α-La is more than four times lower [12].

During the elution with 0.2 and 0.5 M NaCl it could be seen, that all protein eluted at NaCl-concentrations between 0.1 and 0.2 M NaCl. Most probably peak separation can be optimized by adjusting the gradient steps. Instead of 0.2 and 0.5 M the steps have been changed into 0.05, 0.1 and 1.0 M NaCl (see Figure 2). At 0.05 M NaCl α-La and IgG eluted. As expected, BSA eluted within the second peak and β-Lg was a sharp peak at 1.0 M NaCl.

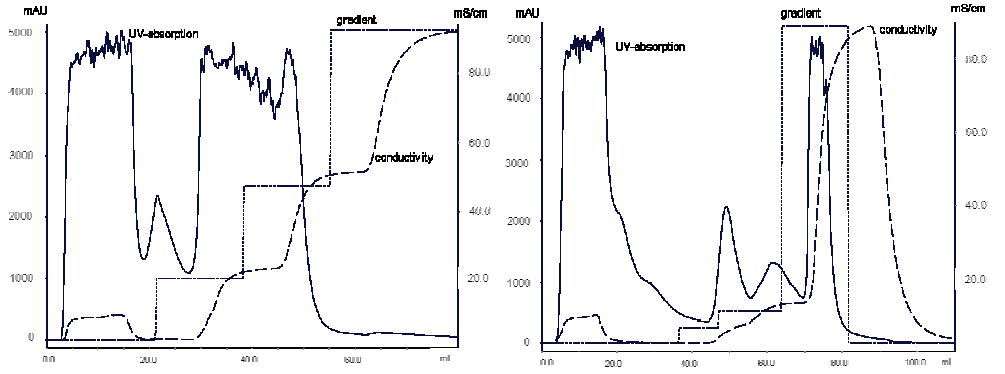

Fig. 2. IEC-chromatograms of runs on Q-MA with NaPhos pH 7. (a) 0.2 and 0.5 M NaCl steps; (b) adjusted gradient with 0.05, 0.1 and 1.0 M NaCl-steps

3.2. Binding of whey proteins to cation exchange membranes

The effluent from Q-MA is collected and served as sample for the S-MA process. It contains α-La, Lf, LPO and IgG. Since positively charged proteins will be bound to the cation exchange membrane pH has to be lower than the IEP of the target proteins. Accordingly, for the experiments with S-MA NaAc buffer with pH-range of 4.0-5.6 (steps of 2.0 pH-units) was chosen. In this range Lf, LPO and IgG are supposed to be positively charged (see Table 1 for IEPs) and adsorb to the stationary phase. At pH > 4.5 α-La is not expected to interact with the anionic ligands due to its neutral or negative net charge.

The results showed that the buffer-pH had a significant influence on the adsorption behaviour of α-La. As expected, all α-La bound to the S-MA at pH 4.0-4.4. Also no other proteins were found in the effluent which implies complete adsorption of all proteins present in the substrate. With increasing pH α-La bound less, at pH 5.0 it did not adsorb at all. Best results regarding the fractionation of bound LPO, Lf and IgG were observed at pH 4.8. The proteins could be eluted separately. At 0.25 M IgG was desorbed from the stationary phase, at 0.35 M LPO eluted and at 1.0 M NaCl an amount of Lf (see Figure 3). These fractions have a high purity of approx. 100%. Contrary to the expectations the majority of α-La was bound. Since it eluted at lowest ionic strength, it was collected together with the effluent so that one α-La fraction was obtained. Anyway, binding of α-La should be avoided, because it unnecessarily occupies binding sites. Furthermore, it leads to a more complicated process since an additional elution step is required for removing α-La from the membrane. Besides the results for α-La also elution of Lf is not as expected. It is found in three different fractions: in the effluent a very small amount, and in fractions eluted a relatively

low and high ionic strength. Therefore, the separation of Lf was not very successful. Even if the Lf-fraction obtained with 1 M NaCl is very pure, only less than 30% of total Lf was recovered in this fraction.

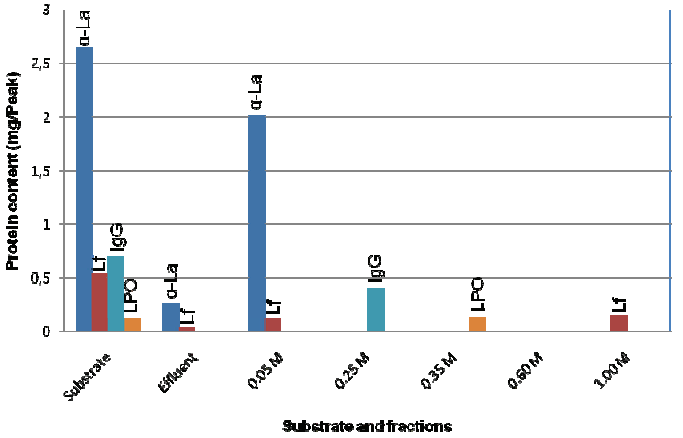

Fig. 3: Protein content in substrate, effluent and elution fractions. Process conditions: NaAc pH 4.8

Based on these results further experiments for improved recovery and purity of Lf and α-La were conducted. The idea was to exploit possible displacement effects for α-La. Until now there is no information available about displacement of α-La on a cation exchange membrane by LPO, Lf or IgG. Anyway, they are likely to occur since binding of those proteins is stronger than that of α-La. This was concluded from a higher ionic strength which is required for desorption of IgG, Lf and LPO. In order to displace α-La the injected amount of protein must be increased to the extent that almost all binding sites are occupied by IgG, Lf and LPO. Different injection volumes between 20 and 100 ml were screened.

Demonstrating the effect of different injection volumes the recovery of α-La and Lf in the effluent and bound fractions is shown in Figure 4. The proportion of bound α-La compared to the unbound part decreases significantly with increasing injection volume. At 20 ml injected sample 20% of α-La is bound and at 100 ml only approx 3% is bound. This is a quite remarkable result, because it seems that a displacement of α-La occurred. Concerning the adsorption of Lf it could be observed that the amount of bound Lf increased with higher injection volumes. At 20 ml approx. 55% Lf bound and at 100 ml 63% was bound. Therefore, the hypothesized effects occurred. Still the recovery of Lf is not really satisfying. One third of total Lf is found in the effluent mixed with α-La and not isolated. However, the amount of Lf is minor compared to the concentration of α-La.

The development of the fractionation of whey proteins on anion and cation exchange membranes has been described. The aim of the study was a development of a two-step fractionation process for all target proteins in acid whey. The final set-up of this process is displayed in Figure 5. The process starts with pH adjustment of pre-treated acid whey to pH 7 and conditioning of the Q-MA. This is done with 0.03 M sodium phosphate buffer at pH 7. Cheese whey is injected and negatively charged proteins are bound, except from α-La, which is displaced from the cationic membrane by β-Lg and BSA. The latter is eluted first at 0.1 M NaCl and β-Lg is desorbed with 1 M NaCl. The effluent from the Q-MA is collected and pH is set to 4.8. At the same time the S-MA is equilibrated with 0.1 M sodium acetate at pH 4.8. Now, the sample is injected and the negatively charged proteins IgG, Lf and LPO interact with the anionic ligands.

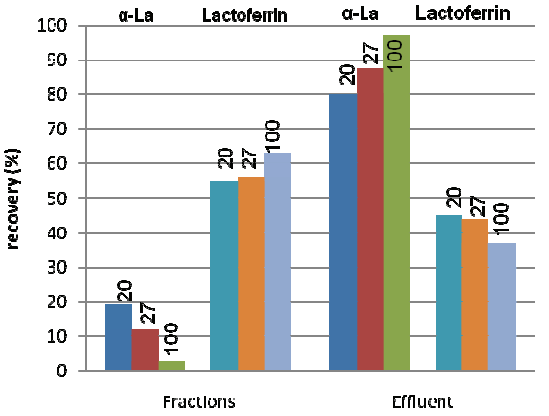

Fig. 4. Influence of the injection volume on the recovery of α-La and Lf in the effluent and the eluted fractions.

In order to avoid adsorption of α-La an excess amount of protein must be injected, so that there are no binding sites left for it. This excess amount is three fold the effluent from Q-MA (100 ml). The effluent from S-Ma contains mainly (88%) α-La and some Lf. Eluted fractions are IgG at 0.25 M NaCl, LPO at 0.35 M NaCl and Lf at 1 M NaCl with a recovery of approx. 63%.

4. Conclusion

In this work it was shown that the fractionation of six target proteins from acid whey with a two-step process is possible. For this purpose the innovative membrane adsorption chromatography has been applied by coupling anion and cation exchange membranes. The recovery and purity of most protein fractions was close to 90% and higher. Only Lactoferrin separation was less successful. The separation was carried out with food grade buffers and elution with ionic strength gradient containing common salt. Therefore, the application of the isolated protein fractions for food applications will not pose any problems. In subsequent studies the practicability of this lab scale process is planned be investigated at pilot scale.

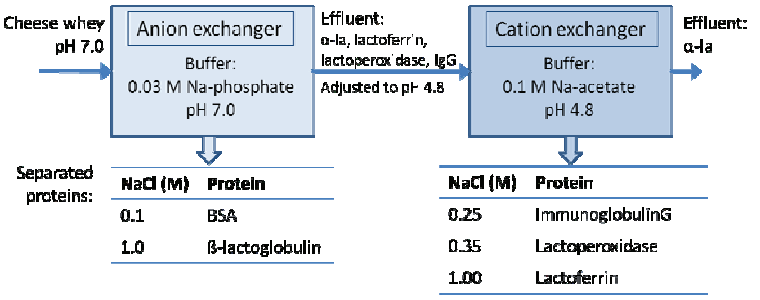

Fig. 5. Schematic presentation of the two-step fractionation process of proteins in acid whey

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